ACTA ABP BIOCHIMICA POLONICA

Paper in Press, No. 14850
Vol. 58, 2011
on-line at: www.actabp.pl

Communication

Effect of *Medicago sativa Mhb*1gene expression on defense response of *Arabidopsis thaliana* plants

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Besides the previously described nitric oxide-detoxification activity we identified new features of class-1 non-symbiotic hemoglobin from *Medicago sativa* (Mhb1). Under *in vitro* conditions, using peroxidase in-gel activity assay, theMhb1 protein was shown to possess also peroxidase-like activity. Due to this activity, in the presence of nitrite and hydrogen peroxide, the protein can mediate autonitration and nitration of other proteins at tyrosine residues, as revealed by tandem mass spectrometry and immune assay approaches. Mhb1 through its multifunctional activities can affect different components of signal transduction cascades operating during plant response to infections. This influence is manifested by Mhb1-mediated selective up-regulation of expression of certain pathogen inducible genes in *Pseudomonas syringae* infected *Arabidopsis thaliana* plants which overproduce Mhb1, as revealed by reverse transcription–quantitative real-time PCR analysis. Changes in expression level of these genes can influence such processes as synthesis of secondary metabolites, protein degradation and biosynthesis of ethylene. They can also result in alteration of pathogen-induced defense response of Mhb1 transgenic plants.

Keywords: nitric oxide, nitrotyrosine, non-symbiotic hemoglobin, pathogen infection, peroxidase activity, *Arabidopsis thaliana*

Received: 28 March, 2011; revised: 10 May, 2011; accepted: 17 JUne, 2011; available on-line: 4 July, 2011

INTRODUCTION

Non-symbiotic hemoglobins (nsHbs) are widely distributed in higher plants. They are organized in two classes differing in affinity for O_2. The nsHbs are involved in nitric oxide ($^{\cdot}NO$) detoxification in plants. Several nsHbs, mainly from class-1, have been shown to possess $^{\cdot}NO$ detoxifying activity similar to that of mammalian hemoglobins (Hbs) (reviewed in Perazzolli *et al*., 2006; Garrocho-Villegas *et al*., 2007; Hoy & Hargrove, 2008). As in, mammals $^{\cdot}NO$ is a signaling molecule which plays a pivotal role in plant responses to biotic and abiotic stresses and also during developmental processes (reviewed in Hong *et al*., 2008; Neill *et al*., 2008; Wilson *et al*., 2008). Expression of genes coding for class-1 nsHbs is induced in plants under hypoxia. It is proposed that the main function of this group of Hbs is protection against nitrosative stress associated with hypoxia. Overexpression of class-1 nsHbs decreases the level of $^{\cdot}NO$ accumulated under hypoxia and increases plant tolerance to this stress (reviewed in Perazzolli *et al*., 2006). Some nsHbs have also been shown to possess peroxidase-like activity similar to that of mammalian Hbs (Sakamoto *et al*., 2004; Violante-Mota *et al*., 2010).

Recent studies showed that nsHbs of class-1 can also be involved in plant response to pathogen infection. However, various nsHbs, despite belonging to the same class, act differently in this process (Perazzolli *et al*., 2004; Qu *et al*., 2005; 2006; Nagata *et al*., 2008). Expression of cotton nsHb *GhHb1* was shown to be significantly induced by infection with the fungal pathogen *Verticillium dahliae* (Qu *et al*., 2005). Overexpression of alfalfa *Mhb1* and *GhHb1* respectively, in transgenic tobacco and *Arabidopsis* plants, increased their pathogen resistance and caused up-regulation of certain defense genes (Seregélyes *et al*., 2003; 2004; Qu *et al*., 2006). In particular, *Mhb1*-transgenic tobacco displayed reduced necrosis formation in response to tobacco necrosis virus (TNV) and *P. syringae* pv. *phaseolicola* (Seregélyes *et al*., 2003). In these plants significant up-regulation of a pathogen-inducible pathogenesis related *PR1a* gene was observed after infection with *P. syringae* pv. *maculicola* (Seregélyes *et al*., 2004). Infected *Mhb1*-transgenic tobacco also displayed a higher level of salicylic acid and superoxide anion ($O_2^{\cdot-}$), molecules involved in plant response to pathogen infection (Seregélyes *et al*., 2003).

In this report we show that *Mhb1*-transgenic *A. thaliana* plants infected with an avirulent strain of *P. syringae* pv. *tomato* display up-regulation of several pathogen-inducible genes. Such impact of Mhb1 on pathogen-inducible gene expression can be connected with various pseudo-enzymatic activities of the Mhb1 protein. Recombinant Mhb1 (rMhb1), besides the previously shown $^{\cdot}NO$ detoxification activity, also displays a peroxidase-like activity. This activity enables rMhb1 in the presence of hydrogen peroxide (H_2O_2) to oxidize nitrite (NO_2^-) to nitrogen dioxide ($^{\cdot}NO_2$) which is a nitrating agent of biomolecules. We demonstrated that rMhb1 undergoes autonitration and also can effect nitration of other protein.

MATERIALS AND METHODS

Purification of recombinant Mhb1. To investigate peroxidase-like activity of Mhb1, amino-terminally His-tagged recombinant Mhb1 protein (rMhb1) was

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Abbreviations: BSA, bovine serum albumin; CBB, Coomassie brilliant blue; DAB, diaminobenzidine; Hbs, hemoglobins; LC-MS-MS/MS, liquid chromatography–tandem mass spectrometry; Mhb1, class-1 non-symbiotic hemoglobin from *Medicago sativa*; nsHbs, non-symbiotic hemoglobins; PR, pathogenesis related proteins; pv., pathovar; ROS, reactive oxygen species; RT-PCR, reverse transcription–PCR; RT-qPCR, reverse transcription–quantitative real-time PCR

overproduced in *Escherichia coli* BL21(DE) strain. The pTrcHis B/Mhb1 plasmid containing full-length *Mhb1* cDNA, described previously, was used (Seregélyes *et al.*, 2004). rMhb1 was purified using a three-step purification process including precipitation with ammonium sulphate, Ni-affinity chromatography, and ion-exchange chromatography. The protein was purified to homogeneity as confirmed by LC-MS-MS/MS analysis. For details see Supplementary Methods at www.actabp.pl (A.1). Purified rMhb1 extract was concentrated to 0.5 μg/μl in phosphate buffered saline (pH 7.4) [0.08 M Na_2HPO_4, 0.02 M NaH_2PO_4, 0.01 M NaCl] using an Amicon concentrator (Amicon Ultra-15 centrifugal filter device, Millipore).

Peroxidase activity assay in gel. Three micrograms of rMhb1 was resolved by 5% native polyacrylamide gel electrophoresis. For detection of peroxidase activity, the gel was immersed for 1 h in 0.1 M potassium phosphate buffer (pH 7.0) containing 2 mM H_2O_2 and 0.1% (w/v) diaminobenzidine (DAB). Alternatively, the gel was immersed for 15 min in 0.1 M potassium phosphate buffer (pH 7.0) containing 2 mM H_2O_2, 0.225 mM *p*-coumaric acid and 1.25 mM luminol. The chemiluminescence signal derived from peroxidase-catalyzed oxidation of luminol was detected using X-ray films (Foton Xs-1N, FOTON). Optionally, potassium cyanide (KCN) was used as an inhibitor at a final concentration of 5 mM. A parallel gel was stained with Coomassie brilliant blue (CBB) for confirmation of the presence of protein.

Peroxidase-mediated nitration of protein tyrosine. rMhb1 (0.5 μg) was incubated in phosphate buffered saline with 1 mM H_2O_2 and 1 mM $NaNO_2$ in a total volume of 10 μl of the reaction mixture at 25 °C for 1 h. Optionally, 2 μg BSA was added. Oxidation of NO_2^- to $^{\cdot}NO_2$ was manifested by nitrotyrosine formation.

Detection of nitrotyrosine by Western blotting. Nitrotyrosine was detected using primary rabbit polyclonal anti-nitrotyrosine antibody diluted 1:1000 (Upstate Biotechnology) and secondary sheep anti-rabbit antibody conjugated with horseradish peroxidase diluted 1:80000 (Sigma). For details see Supplementary Methods at www.actabp.pl (A.2). For confirmation of protein presence a duplicate gel was stained with CBB.

Detection of nitrotyrosine by liquid chromatography–tandem mass spectrometry (LC-MS-MS/MS). Prior to analysis rMhb1 was subjected to digestion with trypsin, reduction and alkylation. The resulting peptide mixture was analyzed by liquid chromatography coupled to an LTQ FT ICR mass spectrometer (Hybrid-2D-Linear Quadrupole Ion Trap–Fourier Transform Ion Cyclotron Resonance Mass Spectrometer, Thermo Electron Corp., San Jose, CA). The acquired raw data were processed by Mascot Distiller followed by Mascot Search (Matrix Science, London, UK) against NCBI non-redundant database. For details see Supplementary Methods www.actabp.pl (A.3).

Construction of transgenic plants. The previously described pROK2/Mhb1 construct containing full-length *Mhb1* cDNA (Seregélyes *et al.*, 2003) was used for transformation of *A. thaliana* L. ecotype Col-0 plants by the floral dipping method (Clough & Bent, 1998). The transgenic line obtained by transformation with an empty vector was used as a control. Plants, were grown in growth chambers as was described by Talarczyk et al. (2002).

Plant infection. Four-week-old transgenic *A. thaliana* plants from F3 generation were infected with *Pseudomonas syringae* pv. *tomato* DC3000 *AvrRPM1* strain at a concentration of 10^6 cfu/ml in 10 mM $MgCl_2$. The whole area of the leaf was infiltrated with the bacterial suspension by pressing the solution into the leaf at the abaxial side with a needleless 1-ml syringe. Control leaves were mock-inoculated with 10 mM $MgCl_2$. Leaves were collected at 0 and 24 h post infection.

Reverse transcription reaction. Total RNA was isolated using the method of Chomczynski and Sacchi, (1987) with modifications for plant RNA (according to an MRC Company protocol) using RNA extraction reagent: 38% (v/v) phenol, 0.8 M guanidine thiocyanate, 0.4 M ammonium thiocyanate, 0.1 M sodium acetate (pH 5.0), 5% (w/v) glycerol. One microgram of RNA after DNase treatment (Promega) was used for the reverse transcription reaction in 20 μl volume with oligo (dT) 18 primer and M-MuLV reverse transcriptase (according to a Fermentas Company protocol) for first-strand cDNA synthesis.

Quantitative real-time PCR (qPCR). Primers from "Primer library for *Arabidopsis* pathogen inducible genes" (Sigma) were used with „SYBR Green JumpStart Taq ReadyMix for Quantitative PCR" (Sigma). Primers corresponding to the following genes were used (gene name is followed by the corresponding genome locus in brackets): *ACS6* (1-aminocyclopropane-1-carboxylate synthase 6 – ACC synthase 6, AT4G11280), *ACT2* (actin 2, AT3G18780), *CML41* (calmodulin-related protein 41, AT3G50770), *MATX* (matrixin family protein with peptidase activity, AT1G24140), *MO2* (monooxygenase 2, AT4G38540), *PAD4* (phytoalexin-deficient 4 protein with triacylglycerol lipase activity, AT3G52430), *PR1* (pathogenesis-related protein 1, AT2G14610), *PR4* (pathogenesis-related protein 4, AT3G04720), *PXMT1* (S-adenosyl-L-methionine-dependent paraxanthine methyltransferase 1, AT1G66700), *RHA1A* (RING-H2 finger protein with ubiquitin-protein ligase activity, AT4G11370), *SDR* (short-chain dehydrogenase/reductase family protein, AT2G47130). Amplification, data acquisition, and data analysis were carried out using GeneAmp 5700 Sequence Detection System (Applied Biosystem). The expression level of the genes, shown in arbitrary units, was estimated by relative quantification process, using *ACT2* gene for normalization. For details see Supplementary Methods at www.actabp.pl (A.4).

RESULTS AND DISCUSSION

Peroxidase-like activity of rMhb1 protein

Mhb1 exhibits peroxidase-like activity similarly to animal Hbs and nsHbs from *A. thaliana* and rice (Bao & Williamson, 1997; Sakamoto *et al.*, 2004; Violante-Mota *et al.*, 2010). The Peroxidases catalyze the oxidation of different substrates in the presence of H_2O_2. Peroxidase-like activity of rMhb1 was studied by in-gel activity assay in the presence of two substrates, DAB and luminol (Fig. 1). The peroxidase-like activity of rMhb1 was detected in the gel with both substrates and colocalized with the CBB-stained protein. This activity was completely abolished by KCN, an inhibitor of peroxidase. This analysis demonstrated that the rMhb1 protein possesses a peroxidase-like activity.

rMhb1-mediated protein nitration

Peroxidases can oxidize different substrates including NO_2^-. In the presence of H_2O_2, they oxidize NO_2^- to $^{\cdot}NO_2$ which can react with biomolecules. In the case of proteins, it causes nitration mainly of tyrosine residues

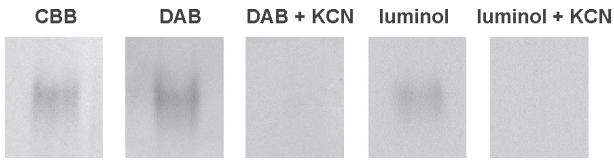

Figure 1. Analysis of peroxidase activity of rMhb1
DAB 0.1% or 1.25 mM luminol were used as substrates and 5 mM KCN as an inhibitor in-gel peroxidase assay of purified rMhb1 (3 µg) after native polyacrylamide gel (5%) electrophoresis. For confirmation of protein presence a parallel gel was stained with CBB.

leading to formation of nitrotyrosine (reviewed in Radi, 2004).

Following the observation that rMhb1 exhibits a peroxidase-like activity, its ability to oxidize NO_2^- and thus to mediate protein nitration was examined. Purified rMhb1 protein was incubated with NO_2^- and H_2O_2 to test its self-nitration ability. Additionally, the reaction was supplemented with a standard nitration target protein, BSA, to test the nitration ability of a coexisting protein. Then, nitration of tyrosine was examined by immunoassay with anti-nitrotyrosine antibodies.

Before evaluation of the results of the nitration test, it should be noted that rMhb1 in the presence of H_2O_2 underwent degradation and oligomerisation as revealed by analysis of a CBB-stained duplicate gel (Fig. 2A–I). NO_2^- partially abolished these H_2O_2-dependent rMhb1 rearrangements (Fig. 2A–I, lanes 4 and 5). This suggests that consumption of H_2O_2 in the peroxidase-catalyzed nitration competes with H_2O_2-dependent degradation.

The observed rMhb1 oligomerisation and degradation could be caused by its oxidative damage, similarly as it was shown the whale myoglobin (Roncone *et al.*, 2005). H_2O_2, besides inducing peroxidase-like activity of the myoglobin, was also shown to promote its oxidative damage. The myoglobin could competitively react with the substrate or undergo autodegradation. Myoglobin oligomers were shown to be generated from tyrosyl radicals and heme-protein cross-linked species, both formed by peroxidase-catalyzed oxidation. Slight H_2O_2-dependent degradation can be also seen in nsHbs from *A. thaliana* tested for their nitrating ability (Sakamoto *et al.*, 2004).

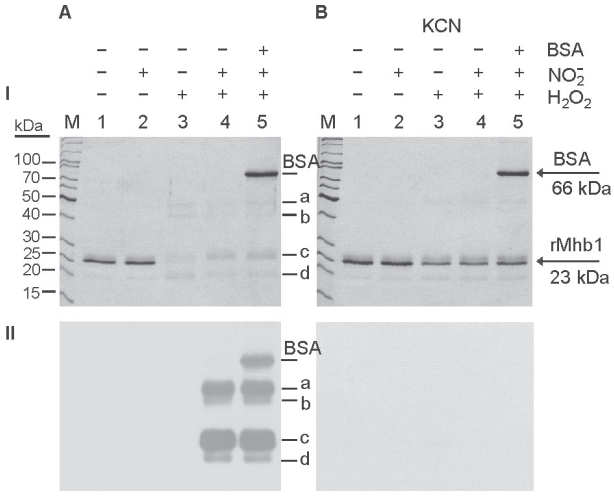

Figure 2. Analysis of nitration of rMhb1 and BSA
rMhb1 (1 µg) and/or BSA (2 µg) were incubated with 1 mM NO_2^- and/or 1 mM H_2O_2 at room temperature for 1 h, and then analyzed for protein nitrotyrosine formation by Western blotting (II). I, duplicate SDS/PAGE gel (15%) stained with CBB; **(A)** analysis of protein nitration mediated by rMhb1; **(B)** corresponding reactions supplemented with 5 mM KCN, M, molecular weight protein marker; a, rMhb1 dimer; b, dimer of degraded rMhb1; c, rMhb1 monomer; d, degraded rMhb1 (around 18 kDa).

Western blot analysis with anti-nitrotyrosine antibodies revealed that incubation of rMhb1 with NO_2^- or H_2O_2 alone did not cause nitration (Fig. 2A–II; lanes 2 and 3). In contrast, incubation of rMhb1 with both substrates simultaneously resulted in its strong nitration (Fig. 2A–II, lane 4). Additionally, all forms of rMhb1 underwent nitration (Fig. 2A–II; lane 4; bands a–d). Co-incubation of rMhb1 with BSA in the presence of NO_2^- and H_2O_2 resulted in nitration of both proteins (Fig. 2A–II, lane 5). This study showed that in the presence of NO_2^- and H_2O_2 rMhb1 can mediate tyrosine nitration of itself and other proteins.

The rMhb1-mediated nitration of BSA was rather less intense than rMhb1 auto-nitration even though BSA was present at a twice higher concentration and possessed 21 tyrosine residues. It is possible that $^{\cdot}NO_2$ produced by rMhb1 is first trapped mainly by neighboring tyrosine residues of rMhb1.

The nitration reaction was completely abolished by KCN, demonstrating that nitrotyrosine formation was dependent on the peroxidase-like activity of rMhb1 (Fig. 2B–II; lanes 4 and 5). Although KCN inhibited protein nitration significantly, it affected H_2O_2-dependent degradation and oligomerisation of rMhb1 only partially (Fig. 2B–I, lanes 3, 4 and 5). This suggests that the H_2O_2 effect on rMhb1 does not fully depend on the peroxidase-like activity of rMhb1. Increased of KCN concentrations (up to 25 mM) did not inhibit the effect of H_2O_2 either (not shown).

Mhb1 contains two tyrosine residues (Tyr107 and Tyr145). LC-MS-MS/MS analysis of rMhb1 after nitration revealed that both tyrosine (Y) residues underwent nitration. In the nitrated sample two different Y-containing peptides were identified with a mass shift of 45 Da relative to the masses in an untreated sample, which is the expected mass associated with the nitro group attachment (not shown). The nitro group in these peptides was mapped to the tyrosine residue. A comparative analysis of m/z (mass to charge ratio) values of daughter ions from corresponding Y-containing peptides in the control and nitrated sample, confirming the occurrence of this modification in rMhb1 subjected to nitration reaction, is presented in Fig. 3. The shift of m/z 45 seen in the Y-containing singly charged (+) daughter ions from the nitrated sample versus the control one confirms the nitration of both tyrosine residues. All the modifications of rMhb1, such as nitration or H_2O_2-mediated oligomerisation and degradation, could influence its activity and function.

The results clearly demonstrate that, due to its peroxidase-like activity Mhb1, is capable of utilizing NO_2^- to produce the nitrating agent $^{\cdot}NO_2$ and thus lead to tyrosine nitration similarly as animal Hbs (Grzelak *et al.*, 2001).

Analysis of pathogen inducible genes in *Mhb1*-transgenic *A. thaliana* plants

Possessing $^{\cdot}NO$-detoxification and peroxidase-like activity Mhb1 can interfere both with $^{\cdot}NO$ and H_2O_2 metabolism. Thus it can contribute to the modulation of plant response to infection, which has been shown to depend on $^{\cdot}NO$ and reactive oxygen species (ROS), including H_2O_2 and $O_2^{\cdot-}$ (reviewed in Hofius *et al.*, 2007; Mur *et al.*, 2008). $^{\cdot}NO$ and ROS are produced in high amounts during infection and they are key elements of plant resistance response. The balance between $^{\cdot}NO$ and

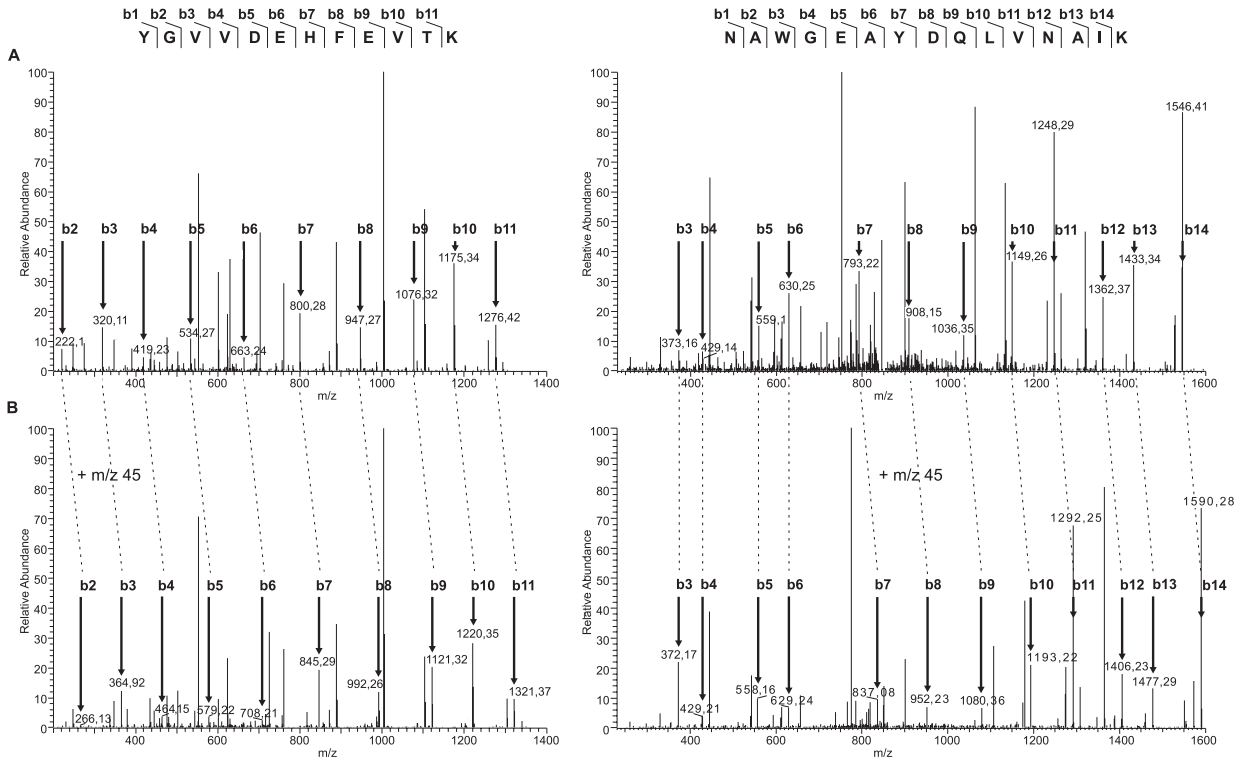

Figure 3. Comparative analysis of LC-MS-MS/MS spectra of Y-containing peptides from control and nitrated sample of rMhb1 protein

rMhb1 (2 µg) was incubated with 1 mM NO_2^- and 1 mM H_2O_2 at room temperature for 1 h. Non-treated rMhb1 protein was used as a control. Prior to mass spectrometry (LC-MS-MS/MS) analysis, protein samples were subjected to standard procedures of trypsin digestion, reduction and alkylation. Figure shows a comparison of fragmentation spectra of two different tyrosine (Y)-containing peptides (YGVVDEHFEVT and NAWGEAYDQLVNAIK) from control sample **(A)** and sample subjected to nitration **(B)**. Peaks in spectra represent daughter ions (b- and y-series ions representing N- and C-terminal parts of a precursor peptide, respectively) which result from fragmentation of precursor peptide during the MS/MS procedure. For clarity, singly (+) charged (z=1) peptide fragments from b-series daughter ions, as the most represented b-ions in the spectra, were considered in the comparative analysis. In the analysis of YGVVDEHFEVT and NAWGEAYDQLVNAIK peptides b2–b11 ions and b3–b14 ions, respectively, were included. By comparing m/z values of b-ions from the Y-containing peptides of two analyzed samples a 45 Da shift can be noted in the Y-containing ions corresponding to the precursor peptides from rMhb1 subjected to nitration reaction.

ROS is an important factor for creating efficient defense response (Delledonne *et al.*, 1998, 2001; de Pinto *et al.*, 2002). They participate in activation of signaling pathways involved in expression of defense genes in plants and also exert a cytotoxic effect on the pathogen and/or infected tissue, thus preventing pathogen spread.

While the ·NO-detoxification activity of plant nsHbs has been shown to lower the ·NO level in nsHb-overexpressing plants (reviewed in Perazzolli *et al.*, 2006), the influence of the peroxidase-like activity of nsHbs on the H_2O_2 level *in planta* is still not clear. There are some contradictory results concerning the role of nsHbs as peroxidases in H_2O_2 metabolism in plants. It was shown that *Arabidopsis* plants expressing class-1 nsHb Ahb1 exhibited a decreased cellular level of H_2O_2 generated during hypoxia (Yang *et al.*, 2005). The observed increased activity of the antioxidant system involved in scavenging of H_2O_2 during hypoxic treatment suggested that this system might be responsible for the low H_2O_2 level in those *Ahb1*-transgenic plants. Overexpression of barley nsHbs in alfalfa plants did not significantly change the level of H_2O_2 a produced under hypoxia stress although an increased activity of the antioxidant system was observed (Igamberdiev *et al.*, 2006).

Violante-Mota *et al.* (2010) suggest that peroxidase-like activity of nsHbs is not of physiological significance *in planta*. Those authors state that it is unlikely that the rice class-1 nsHb they studied, and probably other similar plant nsHbs, function *in vivo* as peroxidases and influence the plant physiology by modulating levels of H_2O_2. They showed that the rice nsHb had a weak peroxidase activity compared with the horseradish peroxidase. However, it partially protected *Escherichia coli* cells expressing recombinant rice nsHb from stress caused by H_2O_2 treatment. Those results do not excludeary influence of nsHbs on H_2O_2 metabolism in plants. The nsHbs can still play a modulating role in this process.

Nevertheless, we showed that overexpression of Mhb1 can disturb the H_2O_2 metabolism *in planta*. Moreover, Mhb1 can thus contribute together with other processes to the synthesis of $\cdot NO_2$; the latter, as a nitrating agent, can influence the activity and stability of biomolecules both in the infected host and the invading pathogen. Protein nitration can constitute a novel level of regulation in signaling cascades operating during plant response to infection. Tyrosine nitration has been shown to increase during defense responses in plants (Saito *et al.*, 2006; Romero-Puertas *et al.*, 2007; Chaki *et al.*, 2009). In mammalian tissues nitration of proteins increases in various pathologies. Nitrotyrosine is commonly detected in infectious and inflammatory diseases (Haddad *et al.*, 1994; Keita *et al.*, 2000; Hickman-Davis *et al.*, 2001; Sittipunt *et*

al., 2001). Protein nitration has been shown in mammalian cells to contribute to cellular signaling in many different processes, including disease development, since it can influence the activity and function of proteins thus modified (reviewed in Monteiro *et al*., 2008; Ischiropoulos, 2009).

Mhb1, by possessing the nitration ability, could participate in protein nitration in plant cells during infection and thus influence the cellular signaling associated with plant defense response.

Mhb1, with its moderate peroxidase-like activity, seems not to be a major player in the metabolism of H_2O_2 or in protein nitration *in planta*. Nevertheless, it can be crucial for these processes due to the interaction of substrates and Mhb1 protein. The peroxidase-like activity of Mhb1 manifests in its autonitration and nitration of selected proteins *in vitro* (recombinant potato 1,3-β-glucanase rGluB20-2, unpublished results), which can modulate their activity. Additionally, the nuclear and cytosolic localization of Mhb1 in plant cells (Seregélyes *et al*., 2000) can contribute to the processes of nitration and metabolism of H_2O_2 or $^{\cdot}NO$ with different kinetics in these two compartments. The nuclear localization of Mhb1 looks especially interesting because its concentration in nucleus is much higher than in the cytosol or appoplast (Seregélyes *et al*., 2000). Thus, the through its multiple activities and dual localization Mhb1 can affect different components of signal transduction cascades operating during plant response to infection.

We asked the question of how increasing these activities *in planta* by Mhb1 overexpression can influence the signaling network of plant defense response manifested by expression of defense genes. We expect that Mhb1 overexpression can influence the processes dependent on molecules which Mhb1 reacts with. The $^{\cdot}NO$-detoxification and peroxidase-like activity of Mhb1 can change the $^{\cdot}NO$/ROS ratio in transgenic plants. Perturbations in the level of these molecules can change the plant response to infection which is dependent on them. Through its nitration activity Mhb1 can also additionally influence the signaling cascades associated with this process by modification of engaged proteins.

We considered one of the elements of the plant immune response — expression of genes associated with the process of plant defense response to infection, in *Mhb1*-transgenic plants. To study the influence of Mhb1 on plant response to pathogen infection, with special emphasis on pathogen-induced gene expression, two independent transgenic *A. thaliana* lines overproducing Mhb1 *versus* a control line were analyzed. Plants were infected with an avirulent strain of *P. syringae* pv. *tomato* DC3000 *AvrRPM1*. A set of 97 pathogen-inducible genes was analyzed using reverse transcription–PCR (RT-PCR). On the basis of results of this analysis (data not shown), ten genes (*ACS6*, *CML41*, *MATX*, *MO2*, *PAD4*, *PR1*, *PR4*, *PXMT1*, *RHA1A*, *SDR*), belonging to different functional groups, with the most evident infection-dependent induction, were chosen. As a reference gene, the *ACT2* housekeeping gene was used. RT-PCR analysis of the expression profiles of the studied genes revealed induction of their expression due to pathogen infection (see Supplementary Data at www.actabp.pl, Fig. B.1). Differences were observed in the expression levels of the studied genes between the *Mhb1*-transgenic and control lines before and after challenge with the pathogen.

To study quantitatively the Mhb1 impact on the expression of the analyzed genes during infection, RT-qPCR analysis was performed. This analysis showed that the expression level of the *ACS6*, *MO2*, *PXMT1* and *RHA1A* gene was higher by, respectively, 60, 35, 70 and 40% (Fig. 4A, B, C, D) in the *Mhb1*-transgenic lines than in the control line.

The higher expression of *ACS6*, *MO2*, *PXMT1* and *RHA1A* genes in the transgenic Mhb1 lines suggests a marked impact of Mhb1 on the transcriptional regulation of these genes. In the case of *MO2* and *PXMT1* include synthesis of secondary metabolites with anti-microbial activity (Aubourg *et al*., 1999; Naoumkina *et al*., 2007) or protein degradation (in the case of *RHA1A*), a common feature of the cell death process occurring during defense response to infection (reviewed in Hofius *et al*., 2007). Particularly interesting was the elevation of expression of the *ACS6* gene encoding a key enzyme in the biosynthesis of ethylene, a plant hormone which participates in plant defense response to infection (reviewed in Hofius *et al*., 2007). Enhancement of the *ACS6* gene expression in the *Mhb1*-transgenic plants can have important implications for the role of this hormone during resistance response.

The expression levels of six other genes, *PR4*, *CML41*, *SDR*, *PR1*, *MATX* and *PAD4*, was similar in both control and *Mhb1*-transgenic lines ($P>0.05$; not shown), which suggests that Mhb1 did not interfere with signaling pathways involved in their transcriptional activation. The unchanged expression of the *PR1* gene in transgenic *Arabidopsis* is in contrast with the up-regulation of the *PR1a* gene in *Mhb1*-transgenic tobacco (Seregélyes *et al*., 2004). It is still possible that Mhb1 can affect their expression differently depending on the pathogen and the host plant species.

The selective Mhb1 impact on the expression of the analyzed genes in transgenic *A.thaliana* plants reflects the fact that the genes may be induced by different cascades. The expression of each of these genes can exhibit different sensitivity to the level of molecules influenced by Mhb1. Mhb1, through its multifunctional pseudo-enzymatic activities and dual localization, can influence dif-

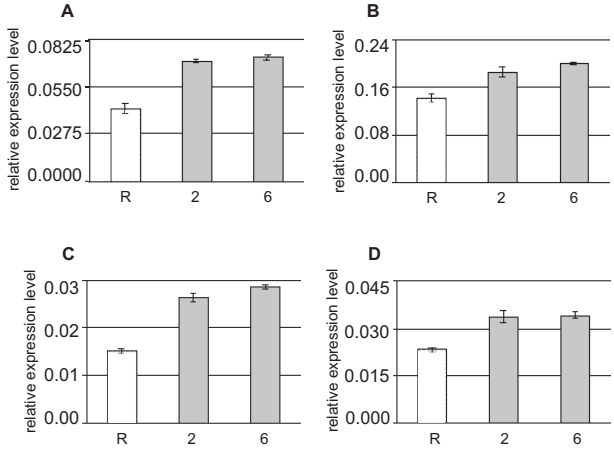

Figure 4. RT-qPCR analysis of expression of pathogen-inducible genes *ACS6*, *MO2*, *PXMT1*, *RHA1A* in *A. thaliana Mhb1*-transgenic lines

RT-qPCR analysis was done for samples collected 24 h post infection with *P. syringae* pv. *tomato* DC3000 *AvrRPM1*. Normalization of expression level of selected genes was performed against the *ACT2* gene. Expression values of *ACT2* were similar in all lines in Student's test, with *P*-value of $P>0.05$ (not shown). Differences in the expression level of the analyzed genes between control (R) and Mhb1 (2, 6) lines were statistically significant ($P<0.05$). The differences between two *Mhb1*-transgenic lines were statistically non-significant ($P>0.05$). **(A)** *ACS6*; **(B)** *MO2*; **(C)** *PXMT1*; **(D)** *RHA1A*.

ferent mechanisms involved in the induction of defense genes and affect them to different extents.

The obtained results support the hypothesis that Mhb1 and other nsHbs can play a role in activation of defense gene expression during infection (Seregélyes *et al.*, 2003; 2004; Qu *et al.*, 2005; 2006). Different pseudo-enzymatic activities of nsHbs can have multiple effects on the plant response to infection.

Appendices

Supplementary Methods (Appendix A), Supplementary Data (Appendix B) at www.actabp.pl.

Acknowledgements

Special thanks to: Dr. C. Seregélyes for providing us the expression vector containing *Mhb1* cDNA for expression of rMhb1 and its subsequent purification, to Prof. D. Dudits for a stimulating discussion, to Dr. K. Witek for help in protein purification, Dr. J. Debski and Dr. J. Sikora for help and technical assistance in the MS/MS data analysis.

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ACTA ABP BIOCHIMICA POLONICA

Supplementary Materials 14850
Vol. 58, 2011
on-line at: www.actabp.pl

Communication

Effect of *Medicago sativa Mhb*1gene expression on defense response of *Arabidopsis thaliana* plants

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Appendix A

Supplementary Methods

A.1. Purification of recombinant Mhb1

Bacterial culture of *E. coli* BL21(DE) strain containing pTrcHis B/Mhb1 plasmid was induced with 0.3 mM isopropyl β-D-thiogalactoside for rMhb1 expression. Subsequently, rMhb1 was purified. All steps of rMhb1 purification were performed at 4°C. The pelleted cells from bacterial culture were resuspended in protein extraction buffer [50 mM Tris-HCl buffer (pH 7.5), 10% (w/v) glycerol, 0.0005% phenylmethylsulfonyl fluoride, 0.05% (v/v) β-mercapthoethanol] and then incubated on ice for 30 min. Cells were disrupted by sonication on ice. The cell lysate was clarified by centrifugation at 12000 x g for 30 min. The resulting supernatant fluid of bacterial lysate, containing soluble protein extract, was precipitated overnight with ammonium sulfate added to a final concentration of 80% (w/v). The precipitate was sedimented by centrifugation at 12000 x g for 30 min and dissolved in buffer A [20 mM sodium phosphate buffer (pH 7.8), 10% (w/v) glycerol] in the same volume as before precipitation. Then, the extract was dialyzed against 200x excess of buffer A (3 changes every 3 h) and applied to a DEAE Sephacel column pre-equilibrated with buffer A. Proteins were eluted from the column with a gradient of 100 – 300 mM NaCl in buffer A. The majority of the rMhb1 protein, in an intensely red fraction, was eluted with 250 mM NaCl in buffer A. Next, rMhb1 was purified by affinity chromatography with Ni-resin according to Qiagen. Purified rMhb1 extract was concentrated to 0.5 $\mu g.\mu L^{-1}$ in phosphate buffered saline (0.08 M Na_2HPO_4, 0.02 M NaH_2PO_4, 0.01 M NaCl) using Amicon concentrator (Amicon Ultra-15 centrifugal filter device, Millipore). Glycerol was added to a final concentration of 5% (w/v). The protein was purified to homogeneity, as confirmed by LC-MS-MS/MS analysis.

A.2. Detection of nitrotyrosine by Western blotting

Proteins after nitration reaction were separated by SDS-PAGE (15%) and electroblotted onto PVDF membrane (Roche). Nitrotyrosine was detected using primary rabbit polyclonal anti-nitrotyrosine antibody (Upstate Biotechnology) diluted 1:1 000 in Tris buffered saline (pH 7.5) [0.01 M Tris-HCl, 0.1 M NaCl], and secondary sheep anti-rabbit antibody conjugated with horseradish peroxidase (Sigma) diluted 1:80 000 in Tris buffered saline. For detection of immuno-complexes reaction buffer for horseradish peroxidase [0.1 M Tris-HCl (pH 8.5), 0.225 mM *p*-coumaric acid, 1.25 mM luminol, 0.0066% H_2O_2] was used, and chemiluminescence emission was detected on X-ray film. For confirmation of protein presence a duplicate gel was stained with CBB.

A.3. Detection of nitrotyrosine by LC-MS-MS/MS

For LC-MS-MS/MS analysis of nitrotyrosine presence in rMhb1, 2 μg of rMhb1 was subjected to nitration reaction as described in *Materials and Methods*. Then, the nitrated sample was dialyzed against phosphate buffered saline using Amicon Ultra-15 centrifugal filter device (Millipore) to remove reactants. Non-treated rMhb1 sample was used as a control. Prior to LC-MS-MS/MS analysis, the protein samples were subjected to digestion overnight with modified trypsin (sequencing Grade Modified Trypsin, Promega), then to reduction with 10 mM dithiothreitol (for 30 min at 56°C), and alkylation with 50 mM iodoacetamide (45 min in darkroom at room temperature). Next, the samples were acidified with 0.1% (w/v) trifluoroacetic acid. The resulting peptide mixture was analyzed by liquid chromatography coupled to LTQ FT ICR mass spectrometer (Hybrid-2D-Linear Quadrupole Ion Trap – Fourier Transform Ion Cyclotron Resonance Mass Spectrometer, Thermo Electron Corp., San Jose, CA) as follows. One of the peptide mixtures was applied to RP-18 precolumn

(LC Packings) using water containing 0.1% (w/v) trifluoroacetic acid as mobile phase and then transferred to nano-HPLC RP-18 column (nanoACQUITY UPLC BEH C18, Waters) using an acetonitrile gradient [0% – 60% (v/v) acetonitrile in 30 min] in the presence of 0.05% formic acid at a flow rate of 150 $nL.min^{-1}$. Column outlet was directly coupled to ion source of LTQ-FT-MS working in the regime of data dependent MS to MS/MS switch. A blank run ensuring lack of cross contamination from previous samples preceded each analysis. Acquired raw data were processed by Mascot Distiller followed by Mascot Search (Matrix Science, London, UK, locally installed http://proteome.pl/mascot) against NCBI non-redundant database. Search parameters for precursor and product ion mass tolerance were respectively ± 40 ppm and ± 0.8 Da, with allowance made for one missed semi-trypsin digestion, fixed modifications of cysteine through carbamidomethylation and variable modification through lysine carbamidomethylation and methionine oxidation.

A.4. qPCR

Primers from "Primer library for *Arabidopsis* pathogen inducible genes" (Sigma) were used with „SYBR Green JumpStart Taq ReadyMix for Quantitative PCR" (Sigma). The reaction mixture contained template cDNA (corresponding to 50 ng of RNA used in the RT reaction), 10 µL 2×SYBR Green JumpStart Taq ReadyMix for Quantitative PCR, 0.4 µL Internal Reference Dye and 400 nM primers in a final volume of 20 µL. PCRs were cycled as follows: 94°C 3 min (94°C 15 s, 65°C 15 s, 72°C 15 s) x 35, 72°C 7 min. Each gene was analyzed in two technical repeats. Amplification, data acquisition, and data analysis were carried out using GeneAmp 5700 Sequence Detection System (Applied Biosystem). For estimation of gene expression level the threshold amplification cycle number (Ct) was used as a quantitative measure of the amount of cDNA product in the sample. It is a fractional cycle number at which the level of fluorescence emitted by the reporter dye incorporated into the DNA

product increases considerably above baseline during PCR. Baseline was assessed arbitrarily. For relative quantification of expression of the genes, logarithmic Ct values were transformed into relative quantities (Q), being geometric raw expression values, using the equation $Q=2^{-Ct}$. Signals from amplification of target genes were normalized against the relative expression level of the *ACT2* gene and expressed as $Q_{target\ gene}/Q_{ACT2}$. The expression level of the target gene was presented as an average value of normalized relative expression and was shown in arbitrary units. Student's test [A.1] in GraphPad InStat Software V2.04a was used for statistical analysis.

References in Appendix A

[A.1] R.R. Sokal, F.J. Rohlf, Biometry: The Principles and Practice of Statistics in Biological Research, Second ed., W.H. Freeman and Company, San Francisco, 1981.

Appendix B

Supplementary Data

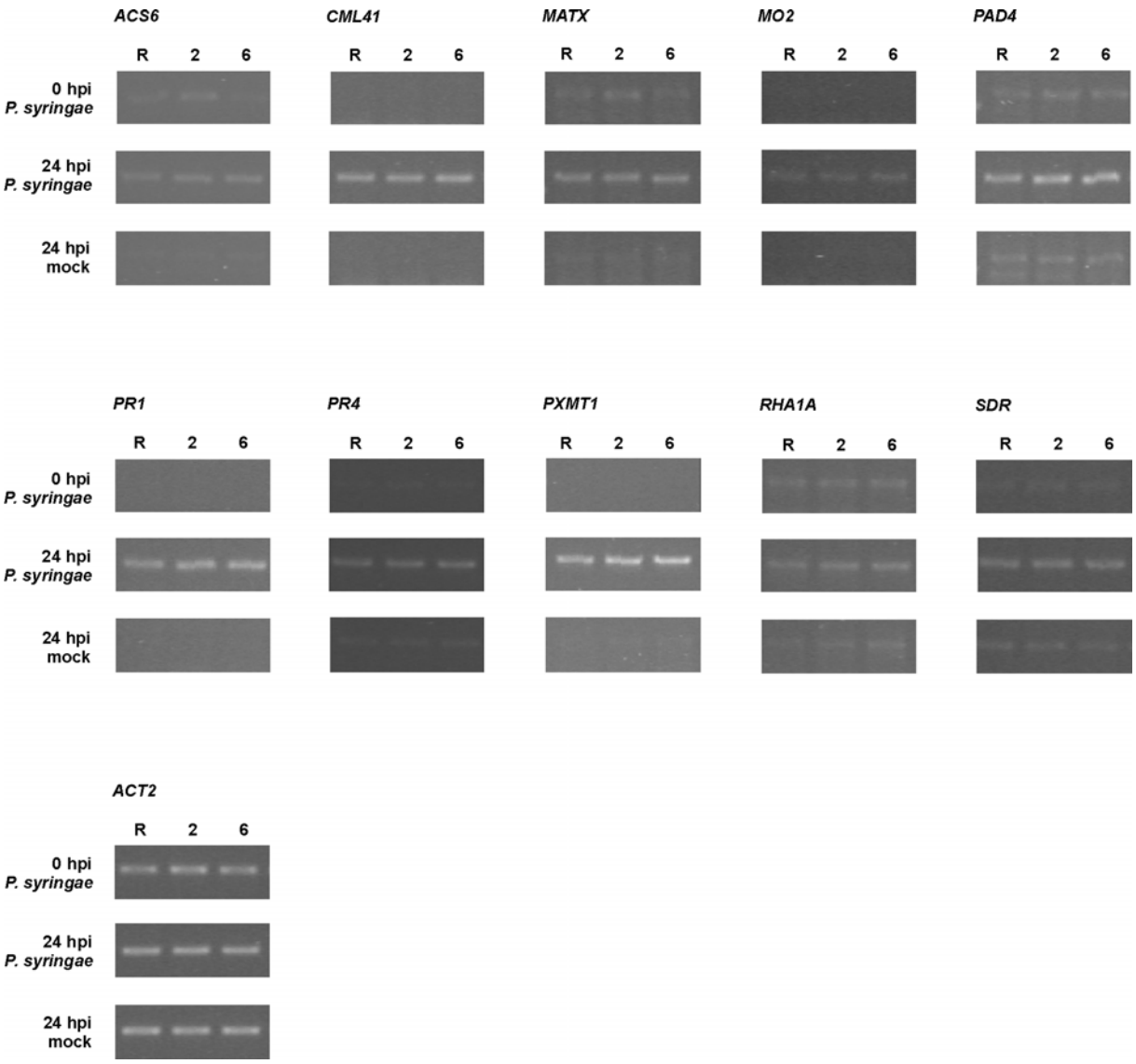

Figure B.1. Semi-quantitative RT-PCR analysis of expression of selected genes in *A. thaliana Mhb1*-transgenic lines.

Analysis was done at 0 and 24 hpi (hours post inoculation) with *P. syringae* pv. *tomato* DC3000 *AvrRPM1* or at 24 h post mock inoculations. RT-PCR products were resolved by electrophoresis in agarose gel stained with ethidium bromide. 2, 6 – *Mhb1*-transgenic lines, R – control line. This graphs represent one of two independent experiments.